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α-Tocopherol Increased Nitric Oxide Synthase Activity in Blood Vessels of Spontaneously Hypertensive Rats

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Antioxidant protection provided by different doses of α-tocopherol was compared by determining nitric oxide synthase (NOS) activity in blood vessels of spontaneously hypertensive rats (SHR) treated with α-tocopherol.

SHR were divided into four groups namely hypertensive control (C), treatment with 17 mg of α-tocopherol/kg diet (α1), 34 mg of α-tocopherol/kg diet (α2), and 170 mg of α-tocopherol/kg diet (α3). Wister Kyoto (WKY) rats were used as normal control (N). Blood pressure were recorded from the tail by physiography every other night for the duration of the study period of 3 months. At the end of the trial, animals were sacrificed. The NOS activity in blood vessels was measured by [^{3}H]arginine radioactive assay and the nitrite concentration in plasma by spectrophotometry at wavelength 554 nm using Greiss reagent.

Analysis of data was done using Student's *t* test and Pearson's correlation. The computer program Statistica was used for all analysis.

Results of our study showed that for all the three α-tocopherol-treated groups, blood pressure was significantly ($P < .001$) reduced compared to the hypertensive control and maximum reduction of blood pressure was shown by the dosage of 34 mg of α-tocopherol/kg diet (C: 209.56 ± 8.47 mm Hg; α2: 128.83 ± 17.13 mm Hg). Also, NOS activity in blood vessels of SHR was significantly lower than WKY rats (N: 1.54 ± 0.26 pmol/mg protein, C: 0.87 ± 0.23 pmol/mg protein; $P < .001$). Although α-tocopherol in doses of α1, α2, and α3 increased the NOS activity in blood vessels, after treatment only that of α2 showed a statistical significance ($P < .01$). Plasma nitrite concentration was significantly reduced in SHR compared to normal WKY rats (N: 54.62 ± 2.96 mol/mL, C: 26.24 ± 2.14 mol/mL; $P < .001$) and accordingly all three groups showed significant improvement in their respective nitrite level ($P < .001$). For all groups, NOS activity and nitrite level showed negative correlation with blood pressure. It was significant for NOS activity in hypertensive control (r = −0.735, $P = .038$), α1 (r = −0.833, $P = .001$), and α2 (r = −0.899, $P = .000$) groups. For plasma nitrite, significant correlation was observed only in group α1 (r = −0.673, $P = .016$) and α2 (r = −0.643, $P = .024$). Only the α2 group showed significant positive correlation (r = 0.777, $P = .003$) between NOS activity and nitrite level.

In conclusion it was found that compared to WKY rats, SHR have lower NOS activity in blood vessels, which upon treatment with antioxidant α-tocopherol increased the NOS activity and concomitantly reduced the blood pressure. There was correlation of lipid peroxide in blood vessels with NOS and nitric oxide, which implies that free radicals may be involved in the pathogenesis of hypertension. Am J Hypertens 1999;12:839–844
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Nitric oxide synthase (NOS) produces nitric oxide (NO) from L-arginine[1] in a calcium- and calmodulin-dependent process.[2] NO is responsible for the acetylcholine-mediated vascular relaxation.[3] NO stimulates soluble guanylate cyclase and there is a concurrent increase in cGMP levels.[4] cGMP inhibits calcium influx and mobilization from intracellular stores,[5] thus facilitating vascular relaxation. Reduced activity of NOS or an accelerated degradation of NO may lead to an impaired vasodilatation, hence increased blood pressure.[6] Oxygen free radicals play a role in the destruction of NO.[7] On the other hand, protein enzymes are much more susceptible to destruction by free radical. Recent studies showed that in hypertension, endothelium-dependent vascular relaxation in essential hypertension was impaired.[8,9] Therefore, it was postulated that increased free radical generation would lead to a reduced activity of NOS or an increased removal of NO in blood vessels, which results in impairment of vascular relaxation. In the mean time, it is reported that L-arginine reduced blood pressure in experimental animals[10] and superoxide dismutase (SOD), a potent free radical scavenger, also reduced blood pressure[11] by increasing the availability of NO.[12] This animal study was undertaken to establish the hypothesis that free radical has a role in the development of blood pressure by influencing NOS/NO and to observe the effects of the natural antioxidant vitamin α-tocopherol on free radical activity in spontaneously hypertensive rats (SHR).

METHODS

Chemicals A basal diet (rat chow) was purchased from Gold Coin Co., Klang, Malaysia. α-Tocopherol (90% purity) used in this study was supplied by Palm Oil Research Institute of Malaysia (PORIM), Bangi, Malaysia. ^{3}[H]-L-arginine (Amersham Radiochemical, Amersham, Buckinghamshire, England), leupeptin, pepstatin, and pefabloc (Boehringer Mannheim, Mannheim, Germany), and all other reagents used were the highest grade commercially available and obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise specified.

Animal treatment Forty-two male SHR, 150 to 200 g, 8 to 10 weeks old, were caged individually and maintained on normal or treated rat chow and water ad libitum for the duration of 3 months (12 weeks). The rats were divided into four groups consisting of the control (C), α-tocopherol treatment with doses 17 mg/kg diet (α1), 34 mg/kg diet (α2), and 170 mg/kg diet (α3). Seventeen age- and weight-matched normal Wister Kyoto (WKY) rats were used as the normal control.

At the end of the study period the rats were killed by cervical dislocation. Blood was taken immediately from the heart. Animals were dissected and approximately 3 cm of the aorta were collected in liquid nitrogen.

Blood pressure monitoring Blood pressure were measured every other night in all animals using the standard tail–cuff method.[13,14] Tails of the animal were occluded with an appropriate size metal tubular tail cuff (7/16 inch) and pulse were detected as the cuff pressure was lowered. The pressure at which the first pulse appears was the measurement of systolic blood pressure. The occluding tubular cuff together with pneumatic pulse transducer (Narco Bio Systems, New York) were connected to an electrophysiograph (Narco Bio Systems, New York) cuff outlet. The rat tails were prewarmed with a lamp before every measurement of blood pressure and the average of three readings was taken as the final reading.

Measurement of NOS and NO Nitric oxide synthase activity was measured in blood vessels with a modified method of Bredt and Snyder[2,15] where production of ^{3}[H]citrulline from ^{3}[H]arginine was measured by a β-scintillation counter. Three centimeters of the aorta was cleaned off its surrounding fat and collected in 1 mL of freshly prepared homogenization mixture (HEPES 20 mmol/L, EDTA 0.05 mmol/L, DTT 1 mmol/L, leupeptin 0.5 μg/mL, pepstatin 0.7 μg/mL, pefabloc 0.5 mmol/L, pH 7.2). Samples were homogenized by Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA Labortechnik, London, UK) and then sonicated three times for 20 sec in Sonicator XL (Heat systems, NY). The homogenized samples were ultracentrifuged at 20,000 g for 25 min. The supernatant (0.34 mL) was mixed with L-[2,3,4,5-^{3}H]arginine (0.6 μCi), L-arginine 50 μmol/L, NADPH 2 mmol/L, $CaCl_2$ 0.45 mmol/L, and calmodulin (10 μg/mL) in a final volume of 0.4 mL and incubated at 37°C for 45 min. N^G-Nitro-L-arginine methyl ester (L-NAME, 1 mmol/L) was used for the determination of blank activity. Immediately after the incubation, assay was terminated by 1 mL of stop buffer (20 mmol/L HEPES, 2 mmol/L EDTA, pH 5.5) and the samples were applied to a 1-mL column of DOWEX (50WX-8, 200–400 dry mash, H^+ form) that were eluted with 2 mL of water. ^{3}[H] was quantified by liquid scintillation in a β-counter. NOS activity were expressed as pmoles of ^{3}[H]citrulline per milligram of protein.

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TABLE 1. COMPARISON OF PARAMETERS OF NORMAL AND SPONTANEOUSLY HYPERTENSIVE RATS (SHR) AT THE END OF 12 WEEKS

Parameters	Normal (n = 17)	SHR (n = 8)	*P* Value
NOS (BV)	1.54 ± 0.26	0.87 ± 0.23	<.001
NO	54.62 ± 2.96	26.24 ± 2.14	<.001*
LP (BV)	0.47 ± 0.17	0.96 ± 0.37	<.001*

NOS, nitric oxide synthase; NO, nitric oxide; BV, blood vessel.
* P < *.05 statistically significant.*
Values Are Mean ± SD.

Nitric oxide was measured as the breakdown product NO_2^-, nitrite levels in the plasma were determined by reacting it with Greiss reagent following the procedure described by Green et al.[16] Sample volume of 500 μL were mixed with 50 μL of 6.5 mol/L HCl and 50 μL of 37.5 mmol/L sulfanilic acid and incubated for 10 min in room temperature. Napthyl ethyline diamine at a volume of 50 μL (12.5 mmol/L) were added and incubated again for 30 min at room temperature. Samples were centrifuged at 1000 *g* for 10 min and the nitrite was quantified spectrophotometrically at wavelength 540 nm against the standards.

Lipid peroxides Lipid peroxides in blood vessels were measured as the thiobarbituric acid reaction product by spectrofluorometry using a well-established procedure[17] and were expressed as nanomoles of MDA equivalent per milligram of protein.

Amount of tissue protein was measured by the procedure described earlier.[18]

Statistical analysis Results obtained were analyzed using ANOVA and Student's *t* test for significant difference. A value of $P < .05$ was considered as significant. Computer program Statistica was used for analysis.

RESULTS

Data analysis showed that SHR have significantly reduced NOS activity in blood vessels, reduced NO in plasma, and increased lipid peroxide in blood vessels (Table 1). After treatment for 12 weeks with different doses of α-tocopherol, data showed that there was a significant reduction in age-related development of blood pressure in all treated groups (Figure 1) compared to SHR control. The best reduction of blood pressure was obtained for the group treated with α-tocopherol 34 mg/kg diet. NOS activity also increased

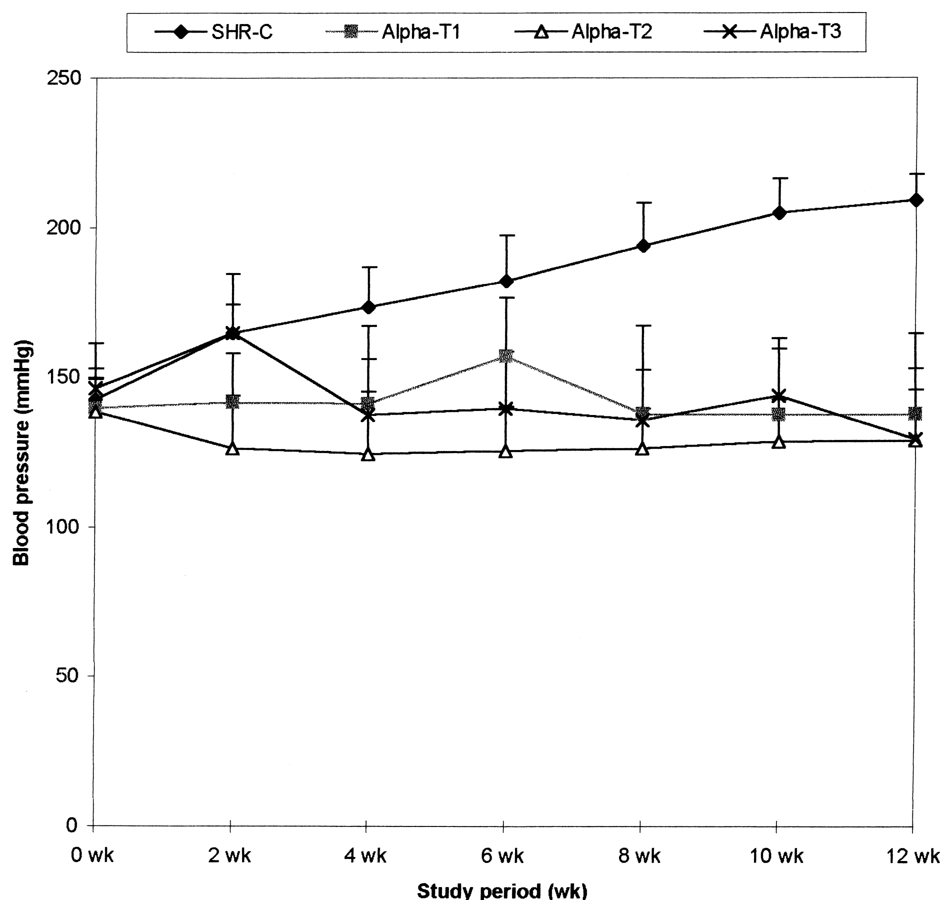

FIGURE 1. *Blood pressure of SHR treated with different doses of α-tococpherol. (SHR-C, SHR control; Alpha-T1, α-tocopherol 17 mg/kg diet; Alpha-T2, α-tocopherol 34 mg/kg diet; Alpha-T3, α-tocopherol 170 mg/kg diet.*

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TABLE 2. COMPARISON OF PARAMETERS OF SHR-CONTROL AND SHR TREATED WITH DIFFERENT DOSES OF α-TOCOPHEROL FOR 12 WEEKS

Parameters	SHR-C	α1 (n = 12)	*P*	α2 (n = 12)	*P*	α3 (n = 10)	*P*
NOS (BV)	0.87 ± 0.23	1.04 ± 0.14	.053	1.29 ± 0.34	.006*	0.89 ± 0.13	.805
NO	26.24 ± 2.14	33.80 ± 3.00	.000*	35.33 ± 2.20	.000*	31.16 ± 1.12	.000*
LP (BV)	0.96 ± 0.37	0.32 ± 0.15	.000*	0.23 ± 0.08	.000*	0.16 ± 0.05	.000*

NOS, nitric oxide synthase; NO, nitric oxide; BV, blood vessel; α1, α-tocopherol 17 mg/kg diet; α2, α-tocopherol 34 mg/kg diet; α3, α-tocopherol 170 mg/kg diet.

* P < *.05 statistically significant.*

Values are mean ± SD.

significantly ($P = .04$) in group α2 compared to SHR control (Table 2). For NO, it was found that plasma nitrite level increased significantly in all the three groups of SHR treated with α-tocopherol compared to SHR control. At the same time lipid peroxide in blood vessels was also significantly reduced in SHR treated with different doses of α-tocopherol (Table 2).

Correlation analysis was done between blood pressure at week 12 and the parameters in all the groups, treated and untreated (Table 3). Results showed that NOS activity and NO have a negative correlation with blood pressure. For NOS, it was significant for groups SHR-C ($P = .038$), α1 ($P = .001$), α2 ($P = .000$). For NO, significant correlations were observed in groups α1 ($P = .016$) and α2 ($P = .024$). On the other hand, lipid peroxide in blood vessels showed a significant positive correlation with blood pressure in groups SHR-C ($P = .003$), α1 ($P = .021$), α2 ($P = .019$), and α3 ($P = .002$). Lipid peroxide in blood vessels was correlated with NOS in blood vessels (Table 4). It gave a negative correlation for all the groups but only significant in the normal ($r = -0.577$, $P = .015$) and α2 ($r = -0.635$, $P = .027$) groups. Lipid peroxide also exhibited a negative correlation with NO in all the groups with a significant correlation in normal ($P = .017$), SHR-C ($P = .033$), and α3 ($P = .038$). A positive correlation was found for NO and NOS in all the groups but was significant only for group α2 ($r = 0.777$, $P = .003$).

DISCUSSION

During the recent years, involvement of free radicals in the pathogenesis of essential hypertension became a major point of research.[11,19] Because the vascular endothelium is the major site for the regulation of vascular tone, increased lipid peroxides found in the blood vessels of SHR of this study supports the hypothesis of free radical involvement.

Acetylcholine-mediated vascular relaxation is manifested through the release of NO in the vascular endothelium,[20] which increases cGMP levels.[21] It was reported earlier that free radicals may reduce the NOS activity, thus decreasing NO. The exact mechanism is not clear, but it may be by direct reduction of NO synthesis from NOS[22] or by interruption of endothelial receptor signal transduction.[23] Besides this, free radicals can act directly on NO and make it less available.[7] In agreement with this suggestion, a reduced NOS activity in the blood vessels of SHR was observed in this study together with reduced NO in plasma. Our findings of a negative correlation between lipid peroxides in blood vessels with the NOS activity and NO further supports the hypothesis.

Treatment with α-tocopherol increased the NOS activity in all treated groups in a variable manner together with increased NO availability. This explains how α-tocopherol prevents the age-related development of increased blood pressure observed in our

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TABLE 3. CORRELATION OF PARAMETERS WITH BLOOD PRESSURE AT 12TH WEEK IN DIFFERENT GROUPS OF SHR TREATED WITH DIFFERENT DOSES OF α-TOCOPHEROL

	SHR-C		α1		α2		α3	
Parameters	r	*P*	r	*P*	r	*P*	r	*P*
NOS (BV)	−0.735	.038*	−0.833	.001*	−0.899	.000*	−0.570	.085
NO	−0.622	.100	−0.674	.016*	−0.643	.024*	−0.496	.145
LP	0.892	.003*	0.653	.021*	0.661	.019*	0.856	.002*

NOS, nitric oxide synthase; NO, nitric oxide; BV, blood vessel; SHR-C, SHR control; α1, α-tocopherol 17 mg/kg diet; α2, α-tocopherol 34 mg/kg diet; α3, α-tocopherol 170 mg/kg diet.

* P < *.05 statistically significant.*

TABLE 4. CORRELATION OF LIPID PEROXIDES IN BLOOD VESSELS WITH OTHER PARAMETERS MEASURED

	Normal (n = 17)		SHR-Control (n = 8)		α1 (n = 12)		α2 (n = 12)		α3 (n = 10)	
Parameters	r Value	*P* Value	r Value	*P* Value	r Value	*P* Value	r Value	*P* Value	r Value	*P* Value
NOS	−0.577	.015*	−0.639	.088	−0.394	.205	−0.635	.027*	−0.335	.344
NO	−0.799	.017*	−0.749	.033*	−0.394	.205	−0.182	.571	−0.659	.038*

NOS, nitric oxide synthase; NO, nitric oxide; α1, α-tocopherol 17 mg/kg diet; α2, α-tocopherol 34 mg/kg diet; α3, α-tocopherol 170 mg/kg diet.
* *P < .05 statistically significant.*

study. From this observation it may be hypothesized that in SHR there is a progressive increase in free radical generation that reduces NOS activity and NO, either directly or by producing lipid peroxides that contribute to the development of high blood pressure. Antioxidant α-tocopherol scavenges this free radical, maintains the NOS activity and availability of NO, and reduces the lipid peroxide formation, thus preventing the development of increased blood pressure.

Correlation observed for NOS and NO with blood pressure in this study gave an insight into their role in the regulation of blood pressure. It is postulated that lipid peroxides have an effect on NOS and NO levels, which influenced the blood pressure. The effect of lipid peroxide on blood pressure was reflected as a positive correlation between the two parameters observed in this study. Further elucidation is required to ascertain whether this influence of lipid peroxide on blood pressure is a direct effect or mediated through NOS and NO.

In conclusion, from this study we found that the age-related development of increased blood pressure in SHR can be attributed to reduced NOS activity with a decreased NO availability due to increased production of free radicals. Treatment with α-tocopherol prevented this development of age-related increased pressure in SHR by increasing the NOS activity in blood vessels and increasing NO levels. This improvement of NOS and NO are mediated through the antioxidant properties of α-tocopherol, where it effectively scavenges the free radicals. Because a positive response was obtained with α-tocopherol treatment of SHR, the involvement of free radicals in the pathogenesis of essential hypertension was ascertained in this study.

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